

Side Population Analysis Using a Violet-Excited Cell Permeable DNA Binding Dye

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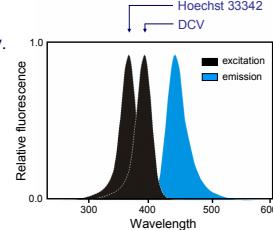
Abstract

Hoechst side population (SP) is a common method for identifying stem cells and early progenitors in both rodent and human hematopoietic tissues. In this technique, the cell-permeable DNA binding dye Hoechst 33342 is loaded into the cell population of interest; stem cells and progenitors subsequently pump this dye out via an ABC membrane pump-dependent mechanism, resulting in a low-fluorescence "tail" (the side population, or SP) when the cells are analyzed by flow cytometry. This population contains stem cells and early progenitors. One significant drawback of this method is the requirement for an ultraviolet laser to excite the Hoechst 33342. Flow cytometers equipped with UV sources are not common and tend to be expensive. Violet laser sources are less expensive and are more common fixtures on benchtop flow cytometers, but have been previously shown to provide insufficient Hoechst dye excitation for good SP resolution.

One possible solution to this problem is to identify additional fluorescent substrates with the same pump specificity as Hoechst 33342, but with better violet excitation characteristics. DyeCycle™ Violet reagent (DCV, manufactured by Molecular Probes Invitrogen) has emission characteristics similar to Hoechst 33342, but with a longer excitation maxima (approximately 390 nm). When this dye is loaded into mouse and human bone marrow or cord blood, a sharply resolved "side population" is also observed, somewhat similar in appearance to that seen with Hoechst 33342. This side population is similar in appearance with both violet and UV excitation. The DCV SP could be inhibited with ABCG2-specific inhibitors like fumetremorgin C. More importantly, simultaneous immunophenotyping with stem cell markers in mouse bone marrow demonstrated that the DCV SP is restricted to the stem cell population (Lin- Sca-1+ c-kit+) population, as is Hoechst SP. These results strongly suggest that DCV efflux identifies the same stem cell population as Hoechst 33342 efflux. Substituting DCV for Hoechst 33342 in the SP technique should therefore allow functional stem cell analysis on flow cytometers with violet lasers.

Hoechst side population (SP) analysis is a critical technique for detecting stem cells and early progenitors. However, the ABCG2 substrate Hoechst 33342 requires UV excitation, not found on many flow cytometers. Violet laser diodes, less expensive and more readily available for flow cytometry, provide a much lower level of Hoechst 33342 excitation than UV sources, often resulting in poor Hoechst SP resolution.

Since violet laser diodes are now common fixtures on flow cytometers, a violet-excited fluorescent substrate with the same membrane pump specificity as Hoechst 33342 would be a very useful tool for stem cell biology. DyeCycle™ Violet reagent (DCV), a cell-permeable DNA binding dye developed by Molecular Probes Invitrogen, has an emission bandwidth similar to Hoechst 33342, but a longer excitation maxima (about 390 nm). This dye was therefore characterized for its ability to identify stem cells and progenitors using both UV and violet excitation sources.

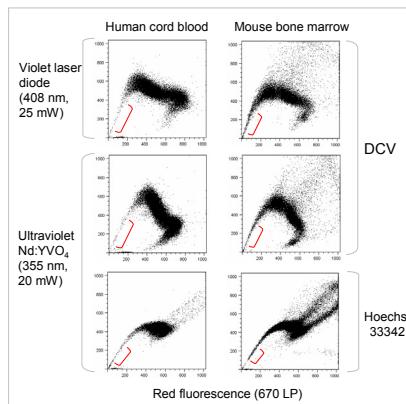


DCV side population

When mouse and human hematopoietic tissues were labeled with DCV (using conditions identical to the original Goodell procedure for Hoechst SP) and analyzed using either UV or violet excitation, a sharply defined "side population" could be visualized using the same blue and red filters normally used for Hoechst 33342. The appearance of the DCV SP was somewhat different than Hoechst SP. However, the DCV SP was very similar in appearance with both UV and violet excitation (unlike Hoechst SP, where violet excitation can lead to a loss in Hoechst SP resolution).

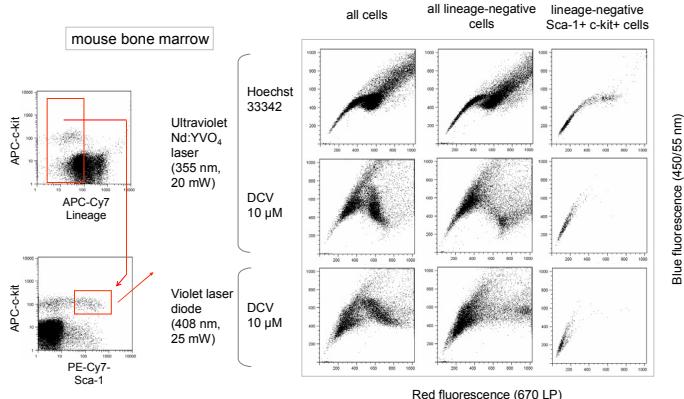
Left, top and middle row: DCV side population analysis in human cord blood (left column) or mouse bone marrow (right column). Excitation was with either a violet laser diode (top row) or a UV laser (middle row). DCV was incubated with cells at 10 µM for 90 minutes at 37°C, washed and stored on ice until analysis.

Left, bottom row: Conventional Hoechst SP analysis in human cord blood and mouse bone marrow, for comparison.



DCV SP is restricted to mouse stem cells and early progenitors (LSKs), as with Hoechst SP

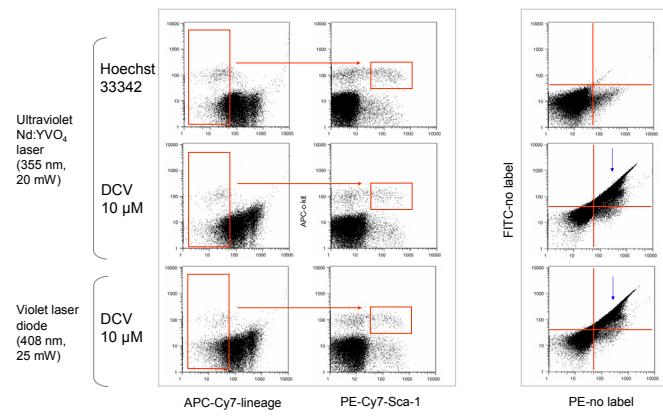
In mouse bone marrow, the Hoechst SP phenotype is strongly associated with Sca-1+ c-kit+ lineage-negative stem cells and early progenitors (referred to as the LSK population). When mouse bone marrow was simultaneously labeled with DCV and the above cell surface markers, the DCV SP phenotype was also strongly correlated with the LSK subpopulation. Again, this analysis could be carried out using either violet or UV lasers.



Above: Simultaneous DCV SP labeling and immunophenotyping with antibodies against mouse Sca-1 (APC), c-kit (PE-Cy7) and a lineage "cocktail" (biotin conjugated followed by APC-Cy7). Cells were analyzed on a BD LSR II, gating on the lineage-negative, Sca-1+ c-kit+ fraction (left scatterplots) with subsequent display of DCV SP in all cells (right scatterplot group, left column), lin-negative cells (middle column) and LSKs (right column). Hoechst SP labeling is shown for comparison (top row).

Other fluorescent characteristics of DCV

Although its excitation was predominantly in the UV to violet, DCV possessed an 488 nm-excited emission "tail" that extended into the green and orange. As a result, a small but noticeable background fluorescence was observable in the fluorescein and PE detection channels during multicolor labeling that was not observable with Hoechst 33342. This effect made FITC and PE labeling for stem cell markers problematic. However, little extraneous emission was observed in the longer red detectors (PE-Cy5.5 and PE-Cy7) or with the red-excited probes (APC and its tandems), making them more readily usable for simultaneous immunolabeling.



Above: Appearance of mouse stem cell immunolabeling in the presence of DCV. The 488 nm long red fluorochromes (such as PE-Cy7) and the red-excited fluorochromes APC and APC-Cy7 were relatively unaffected by DCV fluorescence (left-most scattergrams), but FITC and PE detectors showed considerable overlap (right scattergrams). Hoechst 33342 showed no such effect (top row).

DCV, a violet-excited cell-permeable DNA dye, was therefore able to distinguish a "side population" with the same stem cell / progenitor restriction as Hoechst 33342.